Research Article

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Inhibition of intestinal bile acid absorption improves cholestatic liver and bile duct injury in a mouse model of sclerosing cholangitis

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Background and Aims: Approximately 95% of bile acids (BAs) excreted into bile are reabsorbed in the gut and circulate back to the liver for further biliary secretion. Therefore, pharmacological inhibition of the ileal apical sodium-dependent BA transporter (ASBT/SLC10A2) may protect against BA-mediated cholestatic liver and bile duct injury.

Methods: Eight week old *Mdr2*$^{-/-}$ (*Abcb4*$^{-/-}$) mice (model of cholestatic liver injury and sclerosing cholangitis) received either a diet supplemented with A4250 (0.01% w/w) – a highly potent and selective ASBT inhibitor – or a chow diet. Liver injury was assessed biochemically and histologically after 4 weeks of A4250 treatment. Expression profiles of genes involved in BA homeostasis, inflammation and fibrosis were assessed via RT-PCR from liver and ileum homogenates. Intestinal inflammation was assessed by RNA expression profiling and immunohistochemistry. Bile flow and composition, as well as biliary and fecal BA profiles were analyzed after 1 week of ASBT inhibitor feeding.

Results: A4250 improved sclerosing cholangitis in *Mdr2*$^{-/-}$ mice and significantly reduced serum alanine aminotransferase, alkaline phosphatase and BAs levels, hepatic expression of pro-inflammatory (*Tnf-α, Vcam1*, *Mcp-1*) and pro-fibrogenic (*Col1a1*, *Col1a2*) genes and bile duct proliferation (mRNA and immunohistochemistry for cytokeratin 19 (CK19)). Furthermore, A4250 significantly reduced bile flow and biliary BA output, which correlated with reduced *Bsep* transcription, while *Ntcp* and *Cyp7a1* were induced. Importantly A4250 significantly reduced biliary BA secretion but preserved HCO_3^- and biliary phospholipid secretion resulting in an increased HCO_3^-/BA and PL/BA ratio. In addition, A4250 profoundly increased fecal BA excretion without causing diarrhea and altered BA pool composition, resulting in diminished concentrations of primary BAs tauro-β-muricholic acid and taurocholic acid.

Conclusions: Pharmacological ASBT inhibition attenuates cholestatic liver and bile duct injury by reducing biliary BA concentrations in mice.

Introduction

In the *Mdr2*$^{-/-}$/*Abcb4*$^{-/-}$ mouse model of sclerosing cholangitis, bile duct injury develops as a consequence of defective biliary phospholipid (PL) secretion and subsequent increase of free non-micellar and potentially toxic bile acid (BA) concentrations in bile [1]. As a result, pericholangitis, ductular proliferation and typical onion skin type periductal fibrosis develops spontaneously in this model [2], thereby reflecting central morphological features of chronic cholangiopathies, such as primary sclerosing cholangitis (PSC) [1,3]. Therapeutic options for chronic cholestatic liver diseases and cholangiopathies, particularly such as PSC are limited and low in efficacy [3]. To date, ursodeoxycholic acid (UDCA) is the only approved treatment for cholestatic liver disease [4]. However, its efficacy in patients with PSC and cholestatic liver injury due to mutations in the *MDR3* gene (human orthologue of murine *Mdr2*) is limited [5,6]. Since altered bile composition is an important pathogenic factor for initiation

Keywords: Sodium-dependent BA transporter (ASBT/SLC10A2); Sclerosing; Cholangitis; Liver and bile duct injury.

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and/or progression of cholestatic liver and bile duct injury [7,8] modulation of bile composition arises as an interesting strategy for therapeutic interventions.

Following biliary secretion, BAs undergo enterohepatic circulation, a process where the ileal brush border membrane transporter, the apical sodium-dependent bile salt transporter (ASBT/SLC10A2) [9], mediates the active re-uptake of up to 95% of BAs from terminal ileum and their return back to liver, thus playing an important role in regulating the rate of biliary BA secretion. We, therefore, hypothesized that interruption of BA enterohepatic circulation may markedly reduce biliary concentrations of potentially toxic BAs and exert beneficial therapeutic effects on cholangitis and cholestasis. Hence, *Mdr2*$^{-/-}$ mice as a model of sclerosing cholangitis were treated with the selective and potent ASBT inhibitor A4250. Our study demonstrates that interruption of enterohepatic BA circulation *via* ASBT inhibition improves cholestatic liver and bile duct injury in a mouse model of sclerosing cholangitis, suggesting that ASBT inhibitors may represent a novel and promising treatment for cholangiopathies.

Material and methods

Animals

Mdr2$^{-/-}$ mice (FVB/N background) were obtained from Jackson Laboratory (Bar Habor, ME) and housed in a 12 h light/dark house facility with water and standard chow diet (SSNIFF, Soest, Germany) *ad libitum*.

Feeding protocols

A4250 (a specific ASBT inhibitor) was synthesized by Albireo Pharma (41346 Gothenburg, Sweden). Eight week old male *Mdr2*$^{-/-}$ mice received either control diet or a diet supplemented with 0.01% (w/w) A4250 either for 4 weeks or for 1 week. The 4 week treatment protocol was used for biochemical, molecular and histological data analysis, whereas the 1 week treated mice were subjected to bile flow measurement. The experimental protocols were approved by the local animal care and use committee according to the criteria outlined in the Guide for the care and Use of Laboratory Animals prepared by the U.S. National Academy of Science (National Institutes of Health publication 86-23, revised 1985).

Food intake, body weight/liver weight measurement

Food intake and body weight were controlled weekly. Liver weight (LW) to body weight (BW) % ratio as well as spleen weight (SW) to BW % ratio was calculated.

Routine serum biochemistry

Blood was collected retro-orbitally after 2 weeks and 4 weeks of experimental feeding. Enzymatic assays were used to measure serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) as well as alkaline phosphatase (AP) and bilirubin (Roche Diagnostics, Mannheim, Germany), whereas serum BA levels were measured with the Bile Acid Kit (Ecoline S+ from DiaSys Diagnostic Systems GmbH, Holzheim, Germany) in a Hitachi 917 Analyzer (Boehringer Mannheim, Mannheim, Germany).

Histology

For conventional light microscopy, organs (liver, gallbladder and colon) were fixed in 4% neutral buffered formaldehyde solution for 24 h and embedded in paraffin. Sections were cut 4 μm thick and stained with Hematoxilin and Eosin (H&E) or Sirius Red as described [2]. Quantification of Sirius Red positive area was performed using Image J program (v1.47v; National Institute of Health, Bethesda, Maryland).

Immunohistochemistry

Detection of the cholangiocyte marker cytokeratin (CK) 19 was performed with immunohistochemistry (IHC) as described [10]. To assess colonic inflammation, cell proliferation and glucagon-like peptide 1 (GLP-1) expression, colon slices were stained immunohistochemically for the macrophage marker F4/80, the proliferation marker Ki67 and GLP-1 in addition to conventional H&E staining after 1 week of chow or A4250 feeding. Cell proliferation and macrophage infiltration was quantified by ImageJ software (v1.47v; National Institute of Health).

Hepatic hydroxyproline content

To quantify liver fibrosis, hepatic hydroxyproline was measured from a standardized liver lobe as described previously [11].

Measurement of bile flow and composition

Bile flow and biliary BA profile measurements were performed as described previously [2]. Experimental feeding was initiated in 8 weeks old male *Mdr2*$^{-/-}$ mice. After 7 days of feeding the common bile duct was ligated and the gallbladder was cannulated. After a 10 min equilibration period, bile was collected in preweighted tubes for 30 min. Bile flow was determined gravimetrically and normalized to LW. Biliary PL concentration were measured by Wako Phospholipid Kit and biliary bicarbonate concentrations were measured in a routine laboratory.

Bile acid profiling by ultra-performance liquid chromatography tandem mass-spectrometry

Profiles of murine primary and secondary unconjugated and conjugated C24-BAs in serum (30 μl), bile (15 μl, diluted by 1 ml MeOH), and feces were analyzed as published previously [12] on an Applied Biosystems AB SCIEX QTRAP 5500 platform. Taurine-conjugated tetrahydroxylated BAs were identified from their molecular anion m/z 530, and quantified in relation to d4-taurocholic acid (D4-TCA).

Messenger RNA analysis and Polymerase Chain Reaction (PCR)

RNA isolation from liver and intestine, complementary DNA synthesis and real-time PCR were performed as described previously [13]. Oligonucleotide sequences are available upon request.

Western blot analysis

Protein expression was quantified as described previously [14].

Statistical analysis

Results were evaluated using SPSS V.14.0. Statistical analysis was performed using one-way analysis of variance test followed by student *t* test. Data were reported as means of 5 animals per group ± SD. A p value $\leqslant 0.05$ was considered significant.

Results

Selective ASBT inhibitor A4250 improves cholestatic liver and bile duct injury in Mdr2$^{-/-}$ *mice*

A4250 was well tolerated in *Mdr2*$^{-/-}$ mice and had no impact on animal behavior or body weight after short- or long-term feeding (1 and 4 weeks, respectively; data not shown). In our study we focused on potential changes of cholestatic liver and biliary injury in *Mdr2*$^{-/-}$ mice at 8 weeks of age, a time point when bile duct injury is fully established with pronounced cholestasis [11] in these mice. As determined by liver histology, A4250 ameliorated bile duct injury with pericholangitis and onion skin type fibrosis

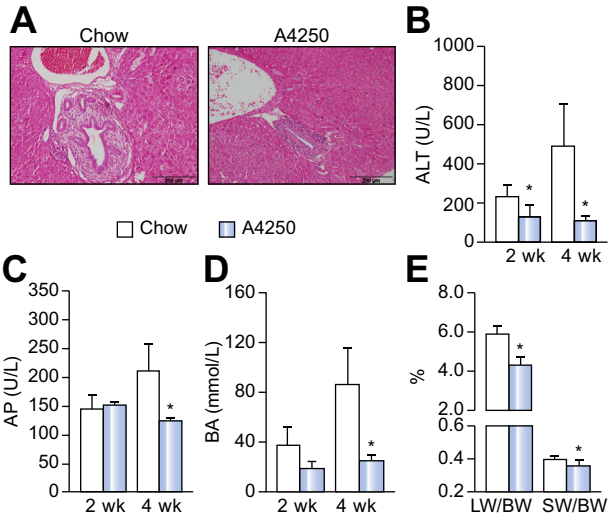

Fig. 1. A4250 improves sclerosing cholangitis and cholestatic liver injury in *Mdr2*$^{-/-}$ mice. (A) Representative liver histology of 12 weeks old chow-fed *Mdr2*$^{-/-}$ mice show typical onion skin type fibrosis. Liver histology of A4250-treated, age-matched *Mdr2*$^{-/-}$ mouse shows improved pericholangitis and onion skin fibrosis after 4 weeks of A4250 feeding. (B, C and D) Serum biochemical parameters of hepatocellular injury (ALT) and cholestasis (AP and BAs) in *Mdr2*$^{-/-}$ mice after 2 and 4 weeks of either chow or A4250-supplemented diet feeding. *Mdr2*$^{-/-}$ mice show increased serum ALT, AP and BA levels, which progresses with age. Compared with chow-fed controls (open bars), serum ALT levels decrease at 2 weeks and AP and BA levels after 4 weeks of A4250 feeding in *Mdr2*$^{-/-}$ mice (blue bars). (E) Compared with age-matched littermates, LW/BW (%) and SW/BW (%) are significantly lower in *Mdr2*$^{-/-}$ mice after 4 weeks of A4250 feeding. Values are means ± SD for n = 5 animals per group. *p <0.05 A4250-fed *Mdr2*$^{-/-}$ *vs.* chow-fed *Mdr2*$^{-/-}$ mice. ALT, alanine aminotransferase; AP, alkaline phosphatase; BAs, bile acids; bd, bile duct; BW, body weight; LW, liver weight; SW, spleen weight.

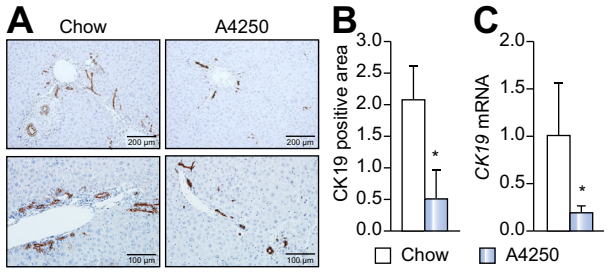

Fig. 2. A4250 reduces bile duct proliferation in *Mdr2*$^{-/-}$ mice. (A) Representative immunohistochemical stainings for cholangiocyte cytoskeleton marker cytokeratin 19 (CK19) (brown) demonstrate reduced number of proliferating bile ducts after A4250 feeding. Portal tract-centered photomicrograph reflects decreased inflammation and onion skin type fibrotic tissue around the large bile duct of A4250-treated *Mdr2*$^{-/-}$ mouse (upper panel). Photomicrographs of the liver lobule reflect decreased periportal lobular extensions of ductular structures after A4250 feeding (lower panel). (B) Computerized analysis (ImageJ) of immunohistochemistry reflects reduced CK19 expression in A4250 treated animals. (C) CK19 mRNA expression is significantly reduced by A4250 in *Mdr2*$^{-/-}$ mice. Gene expression levels are normalized to *36b4* housekeeping gene expression and mean values in chow-fed *Mdr2*$^{-/-}$ mice are set as 1. Results are presented as means ± SD for n = 5 animals per group. *p <0.05 A4250-fed *Mdr2*$^{-/-}$ mice *vs.* chow-fed *Mdr2*$^{-/-}$ mice.

in *Mdr2*$^{-/-}$ mice after 4 weeks of feeding (Fig. 1A). Notably, serum ALT as marker of hepatocellular injury was significantly decreased already after 2 weeks (Fig. 1B), whereas serum markers of cholestasis (AP and BA) were significantly decreased after 4 weeks of A4250 feeding in *Mdr2*$^{-/-}$ mice (Fig. 1C and D). This indicates that longer treatment is required to observe the full impact on biliary injury. In line with serum liver enzymes and BAs, serum bilirubin also slightly but significantly decreased in *Mdr2*$^{-/-}$ mice after A4250 feeding (0.085 ± 0.0 *vs.* 0.16 ± 0.1 mg/dl). Interestingly, serum triglyceride levels remained unchanged, while serum cholesterol increased in *Mdr2*$^{-/-}$ mice after dietary A4250 supplementation (Supplementary Fig. 1). In line with the histological and biochemical findings, liver LW/BW and SW/BW% ratios were significantly decreased in *Mdr2*$^{-/-}$ mice after A4250 treatment (Fig. 1E), reflecting its overall beneficial effects on liver injury. Furthermore, A4250 significantly decreased proliferation of bile ducts (Fig. 2A) as determined by CK19 IHC staining and quantification (Fig. 2B) as well as assessment of mRNA expression (Fig. 2C). Altogether, these findings established a beneficial role of ASBT inhibition on cholestatic liver and bile duct injury in a mouse model of sclerosing cholangitis.

Hepatic inflammation and periductal fibrosis are reduced by A4250 in Mdr2$^{-/-}$ *mice*

Since ongoing inflammation and reactive proliferation of bile ducts in cholangiopathies are associated with development of biliary fibrosis [3]. We next measured mRNA expression of *Tnf-α*, *Mcp-1* and *Vcam-1*, the main pro-inflammatory cytokines involved in the pathogenesis of liver injury in *Mdr2*$^{-/-}$ mice [11] (Fig. 3A) in addition to expression of biliary fibrosis markers such as *Col1a1* and *Col1a2* (Fig. 3B). Interestingly, transcription of pro-fibrogenic genes such as *Col1a1* and *Col1a2* was profoundly reduced in A4250-treated mice (Fig. 3B). Conversely, hepatic hydroxyproline content and α-SMA protein levels did not differ between the groups (Fig. 3C, 3D). In line with inhibited expression of pro-inflammatory cytokines and pro-fibrogenic genes, quantification of Sirius Red staining revealed significantly reduced peribiliary fibrosis and a tendency towards a reduction of overall fibrosis (Fig. 3E, F). Taken together, these data suggest an anti-inflammatory and moderate anti-fibrotic effect of A4250 over a 4 week treatment course in *Mdr2*$^{-/-}$ mice.

A4250 significantly alters bile acid homeostasis in Mdr2$^{-/-}$ *mice*

Importantly, gene expression of *Asbt* as well as basolateral BA transporter *Ost-α* and *Ost-β* in the ileum of *Mdr2*$^{-/-}$ mice remained unchanged after A4250 feeding, whereas expression of *Fgf15*, an important intestinal regulator of BA synthesis [15] and target of intracellular BA sensor FXR [16,17] was profoundly reduced (Fig. 4A), thereby confirming efficient inhibition of ileal BA uptake. In line, A4250 feeding resulted in 3-fold enhanced transcription of the rate limiting enzyme for BA synthesis cholesterol 7 alpha-hydroxylase (*Cyp7a1*) and sinusoidal BA uptake transporter *Ntcp* in liver, while expression of the canalicular BA export pump *Bsep* was reduced (Fig. 4B). In addition, A4250 reduced gene expression of BA detoxifying enzymes *Cyp3a11*, *Ugt1a1* and *Ugt2b5* and sinusoidal export transporter *Mrp3* without significant alterations of sinusoidal exporters *Mrp4*, *Ost-α* and *Ost-β* (Fig. 4B). Collectively, these findings reflect decreased hepatocellular BA load and compensatory changes of hepatic BA homeostasis.

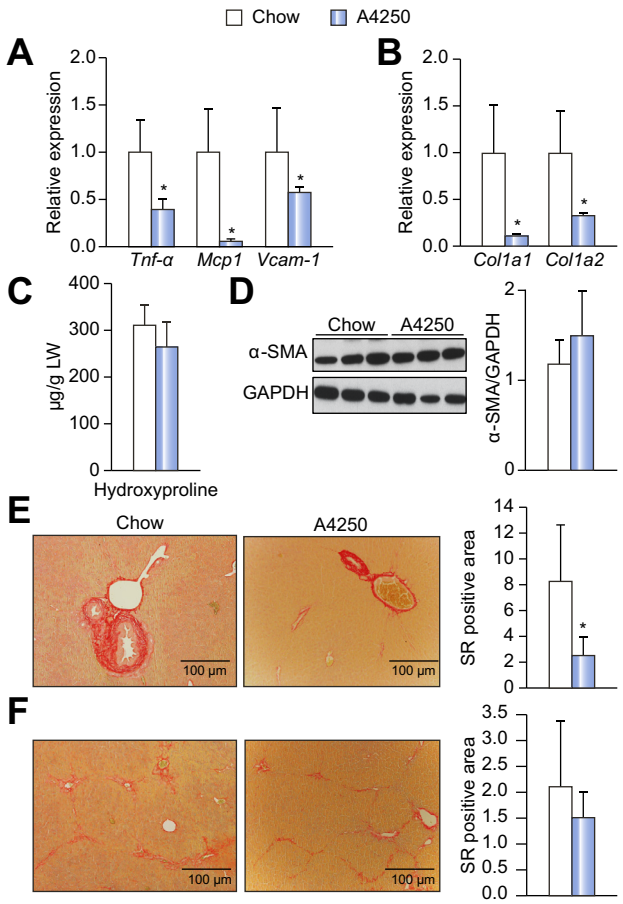

Fig. 3. Hepatic inflammation and fibrosis is decreased in *Mdr2*$^{-/-}$ mice after A4250 feeding. (A) mRNA expression levels of pro-inflammatory markers *Tnfα, Mcp1* and *Vcam-1* were reduced by A4250 (blue bars) compared to chow-fed controls (open bars). (B) A4250 significantly inhibits transcription of hepatic pro-fibrogenic markers *Col1a1* and *Col1a2*. Expression levels are normalized to *36b4* gene expression and mean expression in chow-fed *Mdr2*$^{-/-}$ mice is set as 1. (C) Hepatic hydroxyproline content remained unchanged between the chow- and A4250-fed *Mdr2*$^{-/-}$ mice. (D) α-SMA western blot does not show differences between untreated and treated *Mdr2*$^{-/-}$ mice (E and F) Representative Sirius red staining and quantifications demonstrate typical onion skin-type periductal fibrosis (E) and without pronounced changes in biliary septal fibrosis (F) in both groups. (10× magnification) $^{*}p$ <0.05 A4250-fed *Mdr2*$^{-/-}$ *vs.* chow-fed *Mdr2*$^{-/-}$ mice.

ASBT inhibition reduces biliary BA output and modifies biliary BA composition

A4250 significantly reduced bile flow (Fig. 5A), which was associated with significant reductions of biliary BA concentrations and output (Fig. 5B, C). Furthermore, A4250 significantly altered biliary BA composition by reducing concentrations of primary BAs tauro-β-muricholic acid (TβMCA) and taurocholic acid (TCA) (Fig. 5D). In contrast, concentrations of the secondary BAs taurodeoxycholic acid (TDCA) and taurohyodeoxycholic acid (THDCA) were increased, whereas tauro-ω-muricholic acid (TωMCA) and tauroursodeoxycholic acid (TUDCA) were reduced (Fig. 5E). Of note, unconjugated BAs were not found in bile of chow or A4250-fed mice. Total fecal BA levels were markedly increased in mice subjected to A4250 feeding (Fig. 5F). Of note, anions suggesting the presence of sulphated BAs were not found in feces. Importantly, contrasting diminished BA output, biliary concentrations as well as output of protective HCO_3^- did not differ between the A4250-fed and chow-fed mice (Fig. 5B, C), whereas relative output of HCO_3^- and of PLs was significantly increased in A4250-fed *Mdr2*$^{-/-}$ mice as demonstrated by increased ratios of HCO_3^-/BA and PL/BA (Fig. 5B). Since non-micellar bound free BAs are believed to trigger cholangiopathy in *Mdr2*$^{-/-}$ mice [2,9,11], significant reduction of biliary BA output with simultaneous increase of HCO_3^- proportion may represent an important protective mechanism in toxic bile-mediated liver injury.

A4250 treatment does not promote diarrhea or gut inflammation in Mdr2$^{-/-}$ *mice*

Despite markedly increased total fecal BA levels (Fig. 5F) mice did not develop diarrhea as determined by assessing stool quantity and consistency. As such, we observed no difference in stool weight between control group and A4250 fed mice. (1.30 g stool per day in control mice *vs.* 1.38 g stool in A4250 fed mice). We also addressed whether A4250 promotes colon inflammation. Importantly, conventional histology showed no differences in structure as well as inflammatory cell infiltration of the colonic wall between chow and A4250-fed *Mdr2*$^{-/-}$ mice (Fig. 6A). Furthermore, IHC staining for the macrophage marker F4/80 (data not shown) and the proliferation marker Ki67 (Fig. 6B, E) showed no significant differences between experimental groups. In line with these findings, transcription of pro-inflammatory cytokine *Tnf-α* remained unchanged in the ileum of A4250-treated *Mdr2*$^{-/-}$ mice (Fig. 6D). Since GLP-1 released from enteroendocrine cells in response to increased intestinal BA concentrations may have direct protective effects on cholangiocytes [18], we next addressed whether GLP-1-mediated gut-liver signaling may contribute to A4250-mediated beneficial effects in cholestasis. Notably, GLP-1 IHC did not show differences between control and A4250 fed animals whereas ileal mRNA expression of pre-glucagon was reduced in A4250-fed *Mdr2*$^{-/-}$ mice (Fig. 6C and F), indicating that this mechanism is rather unlikely to contribute to A4250-mediated protection from cholestatic liver and bile duct injury.

A4250 reduces gallbladder size

Since bile homeostasis plays a critical role in gallbladder physiology, we also studied the impact of reduced bile flow and biliary BA output on gallbladder morphology. Interestingly, thickness of gallbladder wall was increased in A4250-fed *Mdr2*$^{-/-}$ mice through increased thickness of *muscularis propria* and number of mucosal folds (Supplementary Fig. 2A, B). Importantly, no increase of inflammatory cell infiltration as reflected by F4/80 staining was detected in *lamina propria*, *muscularis propria* or epithelium (Supplementary Fig. 2C), thereby excluding an inflammation-mediated mechanism of gallbladder wall thickening. Since Fgf15 stimulates gallbladder filling through counteracting cholecystokinin [19], increased gallbladder wall thickness may reflect reduced Fgf15 signaling resulting in a contracted gallbladder in A4250-fed *Mdr2*$^{-/-}$ mice. Importantly, gallbladders of *Mdr2*$^{-/-}$ mice treated with A4250 were not filled with sediments or stones.

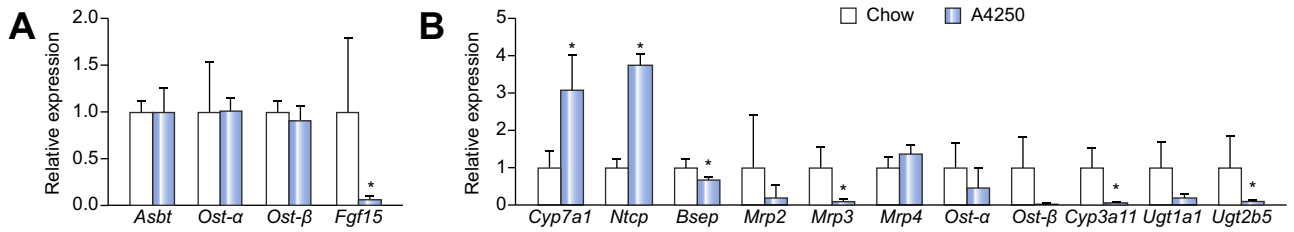

Fig. 4. Impact of A4250 on bile acid homeostasis in *Mdr2*$^{-/-}$ mice. (A) Ileum: Gene expression of ileal *Fgf15* is profoundly reduced, whereas *Asbt*, *Ost-α* and *Ost-β* gene expression remained unchanged by A4250 (blue bars) compared to controls (open bars). (B) Liver: A4250 modifies intrahepatic BA homeostasis by promoting transcription of rate limiting enzyme of bile acid synthesis *Cyp7a1* and basolateral uptake transporter *Ntcp*, with simultaneous repression of canalicular export transporter *Bsep* and sinusoidal export transporter *Mrp3* and inhibition of detoxifying enzymes *Cyp3a11*, *Ugt1a1* and *Ugt2b5*. Expression levels are normalized to *36b4* housekeeping gene and mean values of chow-fed *Mdr2*$^{-/-}$ mice are set as 1. Data are presented as fold change relative to chow-fed mice. Values are presented as means ± SD for n = 5 animals per group. **p* <0.05 A4250-fed *Mdr2*$^{-/-}$ mice *vs.* chow-fed *Mdr2*$^{-/-}$ mice. Bsep, bile salt export pump; Cyp3a11, cytochrome P450; family 3; subfamily a; polypeptide 11; Cyp7α1, cholesterol 7alpha-hydroxylase; Mrp2, multidrug resistance associated protein 2; Mrp3, multidrug resistance associated protein 3; Mrp4, multidrug resistance associated protein 4; Ntcp, Na+/taurocholate cotransporting polypeptide; Ugt1a1, UDP glucuronosyltransferase 1 family; polypeptide A1; Ugt2b5, UDP glucuronosyltransferase 2 family; polypeptide B5.

Discussion

Our study demonstrates that the novel highly potent and selective ASBT inhibitor A4250 decreases cholestatic liver and bile duct injury in the *Mdr2*$^{-/-}$ mouse model of sclerosing cholangitis. After 4 weeks of A4250 treatment, liver histology showed profoundly reduced bile duct injury, pericholangitis and periductal fibrosis (Fig. 1A). Improvement of cholestatic liver injury was further evidenced by reduced serum liver enzymes and BA levels (Fig. 1A–C). At the same time pro-inflammatory mediators as well as bile duct proliferation (both driving biliary fibrosis) [20] were significantly reduced (Figs. 2, 3A). Furthermore, Sirius Red staining and mRNA levels of fibrotic markers (Fig. 3B, E, F) indicate a decrease of fibrosis in *Mdr2*$^{-/-}$ mice treated with the ASBT inhibitor. The finding that hydroxyproline levels were only tendentially reduced in A4250 treated mice could be explained by the fact that all hydroxyproline groups (including those from fragmented collagen) [21] are detected via the photometric measurement, while Sirius Red staining reflects largely the fibrillary collagen deposits in the tissue (type I and III) [21]. Collectively, our data demonstrate that inhibition of ileal BA absorption may have important implications for the treatment of cholangiopathies and cholestatic liver injury.

This improvement in morphological and biochemical features of cholangiopathy of *Mdr2*$^{-/-}$ mice may be explained by the reduction in overall biliary BA output following inhibition of ASBT and subsequent changes in the biliary BA composition (Fig. 5). Interestingly, elevated BA *de novo* synthesis (reflected by increased *Cyp7a1* expression) (Fig. 4B) cannot compensate for the reduction in BA re-uptake. Since HCO_3^- output as well as PL secretion were not changed in response to ASBT inhibitor feeding, the PL/BA ratio (which is critical in micelle formation [22]) as well as the HCO_3^-/BA ratio (important for the protective function of the bicarbonate umbrella [8]) were clearly elevated in A4250 fed *Mdr2*$^{-/-}$ mice (Fig. 5B). Thus, A4250 treatment reduces biliary toxicity in this model by at least two mechanisms. First, the increased PL/BA ratio reduces the concentration of toxic BA monomers. Second, the relative increase of HCO_3^- (the so called bicarbonate umbrella [7,8]) maintains an alkaline milieu which reduces protonation of potentially toxic BA. Notably, other mechanisms such as cholehepatic shunting [23] as well as active induction of ductular HCO_3^- secretion mediated through membrane BA receptor TGR5 may play a critical role in regulating biliary HCO_3^- secretion [24], but do not appear to contribute to the improvement of bile duct injury by A4250 treatment.

Measurement of fecal BAs revealed a considerable increase of BA excretion in A4250 fed animals. BA malabsorption is known to cause diarrhea in humans [25–28]. A recent phase I study reported that A4250 was well tolerated and dose-dependent diarrhea was observed in only 21% of normal volunteers receiving A4250 [29]. Consistent with observations in mice lacking ASBT [30], pharmacological interruption of ileal BA reabsorption by A4250 was accompanied by regular stool behavior without signs of diarrhea in *Mdr2*$^{-/-}$ mice. Importantly, A4250 treated mice also showed no evidence of colonic inflammation. Differences in the incidence of diarrhea between mice and humans may relate to different colonic BA and microbiota composition; e.g. in mice the fecal amounts of chenodeoxycholic acid (considered to be the most potent secretagogue in humans [31,32]) are negligible. Certainly the incidence and severity of diarrhea under A4250 treatment in patients with PSC (and other cholestatic liver diseases) needs to be addressed in future clinical studies.

BA sequestrants represent another approach to interrupt the enterohepatic circulation of BA and are cheap and readily available drugs to inhibit ileal BA absorption. However, they have potential side effects such as constipation, bloating, elevated triglycerides and bad taste and may cause deficiencies of fat soluble vitamins [33]. Interestingly, as proof of principle, cholestyramine has been reported to improve serum parameters of cholestasis in addition to symptoms in PSC [34]. However, colesevelam was not proven to be of sufficient clinical efficacy in cholestasis and showed substantial side effects (such as constipation, flatulence, indigestion and nausea) and poor tolerability in humans [35].

We noted a reduction in gallbladder size with increased wall thickness in mice treated with A4250 (Supplementary Fig. 2). Increased gallbladder emptying has been demonstrated after cholestyramine administration [36,37]. Furthermore, recent findings also demonstrated increased gallbladder contraction when FGF15/19 was absent or reduced [19]. Therefore, increased wall thickness and number of mucosal folds may most likely reflect the non-dispensed state of gallbladder due to abolished *Fgf15* signaling as a result of ASBT inhibition.

In addition to changes in bile composition attenuating toxicity of bile, other intestine-mediated mechanisms may also contribute to A4250-mediated beneficial effects. BAs are key-

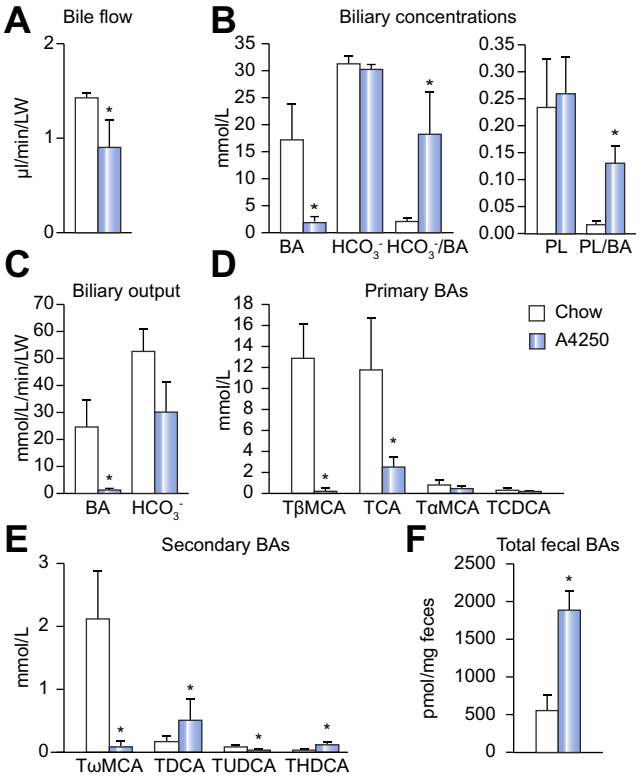

Fig. 5. Effects of A4250 on bile flow and composition. (A) Compared with chow-fed littermates (open bars), bile flow was significantly reduced in A4250-fed *Mdr2*$^{-/-}$ mice (blue bars). (B and C) A4250 reduced concentrations as well as output of biliary bile acids, whereas biliary concentrations and output of protective HCO_3^- remain unchanged. Biliary phospholipids are detected only in trace amounts in *Mdr2*$^{-/-}$ mice and remain unchanged after A4250 feeding. Importantly, HCO_3^-/BA and PL/BA ratios increase in *Mdr2*$^{-/-}$ mice. (D) Concentrations of murine primary bile acids TβMCA and TCA are significantly reduced in bile of A4250-fed *Mdr2*$^{-/-}$ mice. (E) Compared with chow-fed littermates, A4250 significantly reduces biliary concentrations of secondary bile acids TωMCA and TUDCA, while increasing biliary TDCA and THDCA concentrations. (F) Total fecal BA levels are increased in *Mdr2*$^{-/-}$ mice after A4250 feeding. Values are presented as means ± SD for n = 5 animals per group. *p <0.05 A4250-fed *Mdr2*$^{-/-}$ *vs.* chow-fed *Mdr2*$^{-/-}$ mice. BA, bile acids; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TωMCA, tauro-omega-muricholicacid; TαMCA, tauro-alpha-muricholicaicd; TβMCA, tauro-beta-muricholic acid; TLCA, taurolitocholic acid; TDCA, taurodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; THDCA, taurohyodeoxycholic acid.

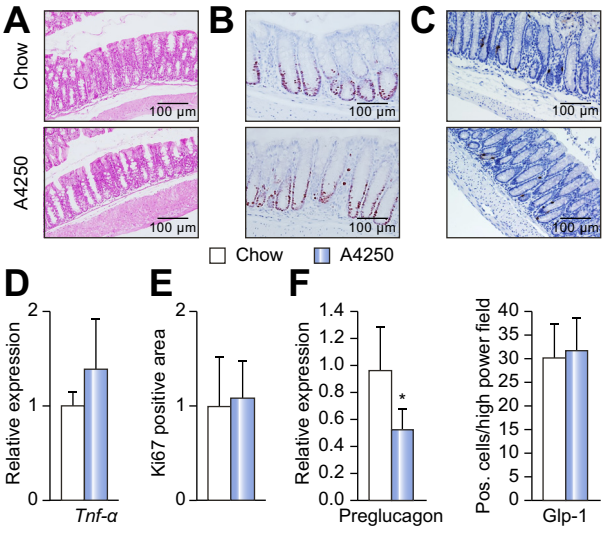

Fig. 6. A4250 treatment does not interfere with colonic inflammation and cell proliferation. (A) A4250 does not alter colon morphology in *Mdr2*$^{-/-}$ mice Histological images of H&E stained colon slices show unchanged colon wall structure in chow – and A4250-fed *Mdr2*$^{-/-}$ mice. (B) Representative histological pictures of Ki67 staining reflect no differences in cell proliferation between untreated and A4250 treated *Mdr2*$^{-/-}$ mice. (C) Representative histological pictures of GLP-1 reflect no differences in expression between control and treated animals. (D) Ileal mRNA expression of inflammatory marker *Tnfα* is not changed in *Mdr2*$^{-/-}$ mice under A4250 feeding. (E) Semiquantitative analysis of Ki67 immunohistochemistry shows no difference between chow-fed and A4250 fed *Mdr2*$^{-/-}$ mice. (F) Ileal mRNA expression of preglucagon (precursor of GLP-1) is even reduced in *Mdr2*$^{-/-}$ mice fed A4250. Computerized quantification of GLP-1 staining does not reveal differences between the different groups. Results are presented as means ± SD for n = 5 animals per group *p <0.05 A4250-fed *Mdr2*$^{-/-}$ *vs.* chow-fed *Mdr2*$^{-/-}$ mice.

regulators of gut microbiota. Dysbiosis has recently been linked to pathogenesis of cholangiopathies such as PSC [38]. Although, potential changes in microbiota composition in *Mdr2*$^{-/-}$ mice still remain to be determined, it is attractive to speculate that blockage of ileal BA transport by A4250 could result in microbiota shifts contributing to part of the therapeutic effects. Therefore, further studies may be justified to explore the impact of ASBT inhibition on changes of gut microbiota in liver disease.

It may be hypothesised that increased exposure of colonic mucosa to BA could alter GLP-1 expression. Entero-endocrine L-cells play an important role in regulating glucose metabolism through GLP-1 [39]. Importantly, BA sequestrants induce GLP-1 precursor pro-glucagon expression and GLP-1 release in a TGR5-dependent manner [39]. Given the fact that A4250 promotes increased BA delivery to the distal parts of gut, secondary BA could induce GLP-1 release through direct and more potent TGR5 activation [39]. Apart from metabolic effects, GLP-1 released from the intestine may enter the liver via portal blood and bind to receptors on cholangiocytes and protect cholangiocytes from BA induced injury/stress by modulating the cholangiocyte adaptive response to cholestasis [40]. Therefore, exendin-4, a GLP-1 receptor agonist, protects cholangiocytes from apoptosis [18,40,41]. However, our data showed unchanged immunostaining of GLP-1 in colon and even reduced mRNA expression of preglucagon (the precursor of GLP-1) in the ileum (Fig. 6E) of A4250 treated mice, consistent with recent findings demonstrating that GLP-1 release is predominantly induced by systemic BAs acting on the basolateral site of the enterocytes [42]. Moreover, it has recently been demonstrated that increased FXR activity in L-cells may even reduce GLP-1 secretion [43].

Our study provided a detailed analysis of intestinal expression of BA-regulated genes such as FGF15/19 and GLP-1 expanding parallel studies on other ASBT inhibitors such as SC-435 [44]. The effects of A4250 on intestinal FXR-signaling and FGF15/19 are in apparent contrast to other promising strategies for treatment of cholangiopathies such as FXR ligands [45]. Fibrates also reduce BA synthesis (via PPARα mediated downregulation of Cyp7a1 and Cyp27) and biliary output despite reduction of FGF-15/19 [46]. One potential benefit for usage of ASBT inhibitors as therapy for cholestatic liver disease may be the reduction of FGF15 expression seen in our A4250 fed animals (Fig. 4A). FGF15/19 is suggested to have potential carcinogenic properties and effects on cholangio-carcinogenesis have to be carefully

considered [47]. It is attractive to speculate, that a combination of such different therapeutic approaches (especially cholehepatic drugs such as conjugation-resistant *nor*UDCA (not undergoing an enterohepatic circulation) [11]) may have complementary therapeutic mechanisms of action.

In conclusion, our study demonstrates that inhibition of ASBT with A4250, a novel, highly potent ASBT inhibitor, reduces bile toxicity by decreasing biliary BA output and concentration, resulting in an improvement of sclerosing cholangitis in Mdr2$^{-/-}$ mice. Therefore, our results suggest that inhibition of ileal BA transporter ASBT may open new avenues for pharmacological treatments of PSC patients.

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Conflict of interest

Michael Trauner and Hanns-Ulrich Marschall have received advisory board fees from Albireo, Sweden. Hans Graffner and Ingrid Påhlman are employees of Albireo, Sweden. Michael Trauner has received research support by Albireo and is listed as co-inventor on a patent on the medical use of norUDCA. The authors who have taken part in this study have declared a relationship with the manufacturers of the drugs involved.

Authors' contributions

AB and CDF: writing of the manuscript, data collection, statistical analysis and interpretation of data

UJL, EK, AW, MS: data collection, critical revision of the manuscript for important intellectual content

CHÖ, EH, IP, HG, PF, GP, HUM: critical revision of the manuscript for important intellectual content

MT: study concept and design, interpretation of data, outlining and revising the manuscript

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2015.10.024.

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